Enhancing endplate permeability to treat or to prevent disc degeneration

Site: University of California, San Francisco	
Site Director: Jeffrey Lotz, PhD	
PI and Co-PI name(s): Aaron Fields (PI) Jeffrey Lotz (Co-PI), Francis Szoka (Co-PI)	**Proposed Budget (including 10% indirects)**: $40,000
PI Phone: (415) 476-0960	**PI E-mail :** aaron.fields@ucsf.edu
CDMI trainee name: TBN	**CDMI trainee title: (e.g. grad student, postdoc, SRA)**
CDMI trainee email: TBN	

Need and Industrial Relevance:
Low back pain is the leading cause of disability and is significantly associated with disc degeneration. Patients with 'discogenic' pain who fail conservative therapy have few treatment options. In particular, there's an urgent need for minimally invasive treatments that halt or reverse early degenerative changes. To that end, newly developed therapies for regenerating a moderately degenerated disc aim to stimulate matrix synthesis using growth factors or augment the nucleus pulposus with stem cells to re-establish nuclear swelling. However, higher matrix synthesis rates and greater cell densities place increased demands on endplate nutrition routes, and since low cartilage endplate (CEP) permeability limits the transport of essential nutrients to disc cells, the long-term efficacy of these new therapies may ultimately require improving CEP permeability. To address this, we propose developing and testing an enzymatic treatment that enhances CEP permeability and nutrient transport. This CEP treatment could be used alone or combined with therapies that regenerate the nucleus pulposus.

Project Aims:
Aim 1: Optimize enzyme attachment and activity of collagenase-lipsomes
To improve CEP permeability, we plan to degrade a controlled amount of collagen matrix with collagenase enzyme, delivered by liposomal carriers. Collagenase will be covalently bonded to custom-made liposomes (~90-100 nm), which are lipid-based vesicles containing an aqueous core. Liposomes are ideal for drug delivery because of their simple construction, tunable drug release properties, excellent syringability, and FDA-approved status. We will compare enzyme attachment and activity for two attachment types: directly to the liposome surface and to poly (ethyleneglycol) (PEG) headgroups.
Aim 2: Evaluate permeability-enhancing activity in human CEP
Liposomes with and without collagenase will be injected into CEP samples harvested from cadaveric lumbar spines. After liposome degradation, CEP permeability enhancement will be evaluated three ways: 1) histologic localization of CEP proteins; 2) measurement of solute diffusivity using fluorescence recovery after photobleaching; 3) assessment of disc cell viability using diffusion chambers.

Methods:
Aim 1: Optimize enzyme attachment and activity of collagenase-liposomes
Collagenase-liposomes. Liposomes will be prepared by sonication and extrusion (Virginia Platt, thesis dissertation, UCSF). Briefly, maleimide lipids (maleimide-DSPE or maleimide-PEG(2000)-DSPE; 5 mol% mPEG(2000); **Fig. 1**) dissolved in organic solvents will be added to glass tubes. After solvent evaporation under vacuum, lipid films will be rehydrated in HEPES buffer with intermittent vortexing and sonication under argon for 10 min at 23°C to form vesicles. Liposome size will be reduced by serial

extrusion through polycarbonate membranes. Collagenase enzyme (2.0 mg/ml in reaction buffer) will be added to the liposomes at 1:10 molar ratio of collagenase to maleimide. After 4 hr reaction time at 21°C, excess free cysteine (10 µL of a 20 mg/mL solution of L-cysteine in PBS) will be added to block unreacted maleimides and the mixture will be incubated overnight at 4°C.

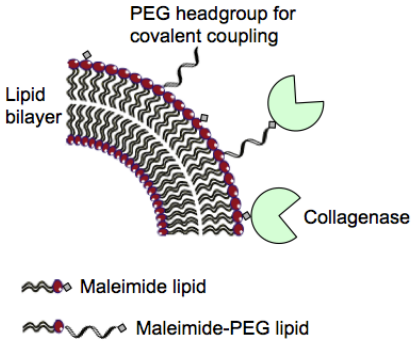

Fig. 1. Collagenase will be conjugated to maleimide-liposomes by covalent attachment to the end of PEG headgroups or covalent attachment directly to the liposome surface.

Collagenase attachment. The attachment efficiency of collagenase to the liposome surface and to mPEG(2000)-DSPE will be measured using the size exclusion method (Virginia Platt, thesis dissertation, UCSF). Briefly, the amount of collagenase associated with liposomes will be determined by comparing liposome-conjugated protein versus total protein in fractions from a size exclusion column. Fractions will be assayed for protein concentration using a Bradford protein assay. Liposome concentration in each fraction will be calculated from FITC-DSPE incorporated into the liposomes (0.1 mol%), referenced to a standard curve generated with known liposome concentrations.

Enzyme activity. To determine if collagenase activity is affected by liposome attachment, the activity of the liposome-bound collagenase will be compared to the activity curve of unbound collagenase. Collagen gels (3 mg/ml; Advanced BioMatrix) will be prepared according to manufacturer's guidelines. Collagenase-liposomes and unbound collagenase will be allowed to degrade the collagen gels for 30 min, 1 hr and 2 hr at 37°C. Activity curves will be determined by measuring the amount of collagen remaining in the gels using a hydroxyproline assay. The attachment method (direct vs. PEG) that yields the highest attachment efficiency and enzyme activity will be used in Aim 2.

Aim 2: Evaluate permeability-enhancing activity

Cadaveric spines. Twelve intact endplates will be obtained from fresh human lumbar spines procured from the UCSF Willed Body Program. To ensure we collect CEP samples with a range of compositions, 4 endplates each will be harvested from discs having MRI Pfirrmann grades II, III, and IV. Four CEP samples will be obtained from the central region of each intact endplate (**Table**).

	Control No collagenase	*Treated* Collagenase
Low conc.	12	12
High conc.	12	12

Enzymatic treatment. Liposomes with and without collagenase will be injected into the CEP samples with low (0.1% total lipid by sample weight) and high concentrations (0.5% total lipid by sample weight). CEP samples will be incubated for 48 hours at 37°C.

FRAP. After incubation, CEP samples will be immersed (48 hr at 4°C) in tracer solution (sodium fluorescein, 376 Da; 0.1 mg/ml). FRAP will be performed with a Leica SP5 laser confocal microscope. Photobleaching (100% power for 4 frames; 6% AOTF pre-/post-bleach) will be performed at >3 spots/sample (25 µm bleach radius). Fluorescence intensity in the bleached spot will be calculated for each image acquired during recovery. Solute diffusivity D will be calculated using the Axelrod method (**Fig. 2**).

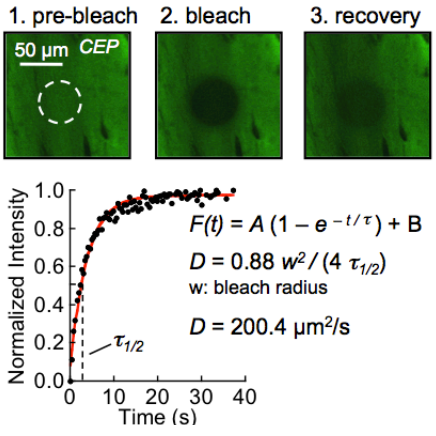

Fig. 2. Following treatment, FRAP will be used to measure the diffusivity, *D*, of a fluorescent tracer in the CEP. After incubation (pre-bleach), a laser is focused on the tissue (bleach). *D* is calculated by curve-fitting the fluorescence intensity during recovery.

Diffusion chambers. Permeability enhancement will also be assessed using diffusion chambers. After treatment, a portion of each CEP sample will be cryostat-sectioned into 170 µm-thick strips, denuded of cells, and rinsed with streptomycin. Nucleus pulposus cells obtained from 28 month-old bovine coccygeal discs will be suspended in media and mixed with low gelling temperature agarose (type VII, Sigma) to give a concentration of 1% agarose and a cell density of 4 million cells/ml. Suspensions will be loaded into the chambers, allowed to gel, and immersed in media (DMEM with glucose, 6% FCS and NaCl). Chambers will be incubated for 3 days under 5%/21% CO2/O2 at 37°C. After incubation, cell viability will be assessed using Live/Dead assays (Invitrogen). Briefly, PBS containing 2 µmol/L of calcein-AM (ex/em: 494/517 nm; fluoresces green in live cells) and 4 µmol/L of ethidium homodimer-1 (ex/em: 528/617 nm; fluoresces red in dead cells) will be pipetted onto the gels. After incubation (30 min, 37°C), gels will be imaged using a fluorescence microscope. Photographs will be taken every 0.5 mm across the chambers. Chambers will be scanned across 3x and the number of live/dead cells (counted using image analysis software) will be averaged at each position in order to determine the viable distance (**Fig. 3**).

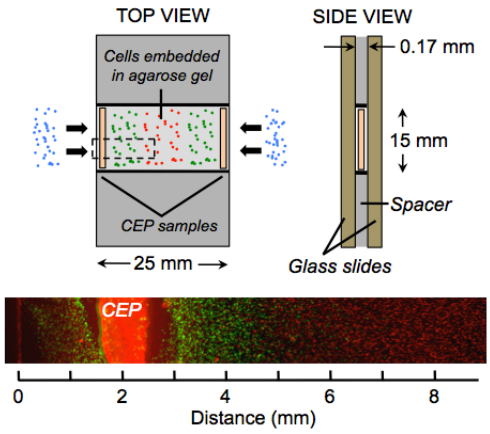

Fig. 3. Diffusion chambers mimic the diffusion-limited environment of the disc *in vivo*. Disc cells in agarose gel are loaded into the chambers. Nutrients (blue) diffuse from medium through the CEP samples at the open sides of the chamber. Live cells fluoresce green; dead cells red. The viable distance depends on CEP permeability (affects nutrient supply) and cell density (affects nutrient demand). The micrograph is from one side of a chamber (see dashed box) incubated with 4 million cells/ml and CEP samples from a 65 year-old donor. Viable distance = 4.5 mm.

Histology. Paraffin histology will be used to evaluate protein depletion. After CEP samples are sectioned for the diffusion chambers, neighboring regions will be fixed, paraffin-embedded and sectioned. Sections will be stained with Safranin-O to assess collagen and proteoglycan distribution.

Milestones:

- Aim 1: Comparison of collagenase-liposomes and collagenase-PEG liposomes – Jan 31, 2016
- Aim 2: Evaluation of permeability enhancement – May 31, 2016
- Finish data analysis – September 30, 2016

Deliverables:

Quarterly presentation updates:

- *December 2015 – conference call*
- *Spring 2015 – Spring Symposium @ UT (conference call option for UCSF teams)*
- *June 2015 – conference call*
- *September 2015 – Fall Symposium @ UCSF (conference call option for UT teams)*

Final written report including results - October 31, 2016

Specific work product (protocols for liposome preparation and results from Aim 1 & Aim 2 experiments)

General Budget Outline:

Personnel	$ 25,700
Supplies	$ 3,864
Specimens	$ 4,000
Imaging	$ 2,800
Total Direct	$ 36,364
Indirects (10%)	$ 3,636
Total	$ 40,000

Start Date:
October 1, 2015

End Date:
September 30, 2016